On the Evolution of Nonribosomal Peptide Synthetase Gene Clusters in Cyanobacteria

by

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LIST OF PAPERS

Paper I:

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Paper II:

Tooming-Klunderud, A., Fewer, D., Rohrlack, T., Jokela, J., Rouhiainen, L., Sivonen, K., Kristensen, T. and K.S. Jakobsen. Microcystin synthetase *mcyABC* operon adenylation domains from three cyanobacterial genera: recombination and selectional forces in naturally occurring strains. Manuscript.

Paper III:

Fewer, D., Tooming-Klunderud, A., Jokela, J., Rouhiainen, L., Kristensen, T., Rohrlack, T., Jakobsen, K.S. and K. Sivonen. An in-frame deletion in the N-methyltransferase domain of the microcystin synthetase gene *mcyA* in strains of genus *Anabaena*. Submitted.

Paper IV:

Tooming-Klunderud, A., Rohrlack, T., Shalchian-Tabrizi, K., Kristensen, T. and K.S. Jakobsen (2007). Structural analysis of a non-ribosomal halogenated cyclic peptide and its putative operon from *Microcystis*: implications for evolution of cyanopeptolins. Microbiology 153, 1382–1393.

Paper V:

Rounge, T.B., Rohrlack, T., Tooming-Klunderud, A., Kristensen, T. and K.S. Jakobsen. (2007). Comparison of cyanopeptolin genes in *Planktothrix*, *Microcystis* and *Anabaena*: evidence for independent evolution within each genus. Applied and Environmental Microbiology, Vol. 73, No. 22, 7322-7330

SUMMARY

Cyanobacteria from genera *Anabaena*, *Microcystis* and *Planktothrix* produce a wide selection of nonribosomal peptides, e.g. microcystins and cyanopeptolins. Nonribosomal peptides are produced by peptide synthetases or polyketide synthase/peptide synthetase hybrids.

In this study, microcystin and cyanopeptolin synthetase gene clusters were analyzed in *Anabaena*, *Microcystis* and *Planktothrix*. Cyanopeptolin synthetase gene clusters were characterized from *Microcystis* and *Planktothrix*. Comparison of these two gene clusters with a previously described cyanopeptolin synthetase gene cluster from *Anabaena* shows similar gene and domain arrangements, while adenylation domains and tailoring domains vary. This suggests independent loss and gain of tailoring domains within each genus as major events leading to the present diversity. Although recombination clearly has been a major factor in the evolution of nonribosomal peptide synthetases, no clear evidence of recombination involving transfer of genetic material across genera was found, neither within the the cyanopeptolin gene clusters nor between the cyanopeptolin and microcystin gene clusters.

Microcystin synthetase genes were investigated in a number of naturally occurring *Anabaena*, *Microcystis* and *Planktothrix* strains. Production of solely unmethylated microcystin isoforms correlated either with lack of the *N*-methyltransferase domain in *Anabaena* strains, or, in *Microcystis,* with point mutations in functionally important sites of the domain. The analysis of adenylation domains encoded by *mcyB* and *mcyC* in the three genera revealed group-specific differences in recombination rates and selectional forces. Analysis of regions flanking the *mcy* gene cluster in *Microcystis* spp. revealed that the genomic location of the *mcy* gene cluster is the same in all strains examined.

ABBREVIATIONS

Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
A-domain	adenylation domain
Ahp	3-amino-6-hydroxy-2-piperidone
apdA-F	genes encoding biosynthesis of anabaenopeptilides in *Anabaena*
ApdA-F	proteins coded by the corresponding genes *apd*A-F
C-domain	condensation domain
Dhb	2-amino-2-butenoic acid
Dha	dehydro-alanine
DNA	deoxyribonucleic acid
E-domain	epimerization domain
HGT	horizontal gene transfer
kb	kilobase(s) = 1000 base pairs
LC/MS/MS	liquid chromatography with mass spectrometric detection
MALDI-TOF-MS	matrix-assisted laser desorption ionisation time of flight mass spectrometry
mcyA-J	genes encoding the microcystin synthetase enzyme complex
McyA-J	proteins coded by the corresponding genes *mcy*A-J
McyA1	first module of McyA
mcyB1	segment of *mcyB1* gene encoding the first module
McyB1	first module of McyB
MC-LR	leucine and arginine in the positions of X and Z of microcystin
MC-RR	arginine and arginine in the positions of X and Z of microcystin
Mdha	*N*-methyl-dehydroalanine
mPro	methyl-proline
ndaA-I	genes encoding the nodularin synthetase enzyme complex
NdaA-I	proteins coded by the corresponding genes *ndaA-I*
NMT-domain	*N*-methyltransferase domain
NRPS	nonribosomal peptide synthetase
ORF	open reading frame
PKS	polyketide synthase
PCR	polymerase chain reaction
sp.	species
T-domain	thiolation domain, alco called PCP
TE	thioesterase

1. INTRODUCTION

1.1 Cyanobacteria

Cyanobacteria are photoautotrophic organisms that comprise a single phylogenetic group within the domain *Bacteria* (Castenholz, 2001). They have photosystems I and II and use water as an electron donor for photosynthetic carbon dioxide reduction. Several cyanobacteria may carry out anoxygenic photosynthesis as well, using only photosystem I, if electron donors such as sulfide are present (Madigan *et al*., 2003). Cyanobacteria are thought to have existed as long as 3500 million years ago, based on documented fossil records (Schopf, 2000). However, DNA-based evidence suggests that cyanobacteria appeared more recently, about 2600 million years ago (Hedges *et al*., 2001).

Cyanobacteria are natural components of all kinds of aquatic habitats. In lakes, they occur as plankton, but also tightly or loosely attached to surfaces of plants, rocks, and sediments. Most cyanobacterial blooms result from proliferation of planktonic cyanobacteria under favorable environmental conditions. In addition, some cyanobacteria are capable of living as terrestrial organisms on rocks and soil and are able to form symbiotic associations with plants, fungi, and animals (Whitton and Potts, 2000). Cyanobacteria show wide ecological tolerance to temperature, light, and desiccation, and possess many characteristics and adaptations that explain their survival, dispersal, and success.

1.2 Cyanobacterial peptides.

Cyanobacteria produce a high number of secondary metabolites and more than 600 peptides and peptidic metabolites have been described from various taxa so far (for a review see Namikoshi and Rinehart, 1996; Welker and von Döhren, 2006). The structural diversity of these peptides is remarkable large, partly due to incorporation of a large number of unusual and modified residues like D-amino acids, β-amino acids as well as variety of hydroxy acids and *N*-methylated acids. Welker and von Döhren (2006) organized the cyanobacterial peptides into seven classes based on the molecular structures.

The best known peptides are the cyclic hepatotoxic microcystins (Figure 1A) and nodularins, which contain the unique amino acid, 3-amino-9-methoxy-2, 6, 8,-trimethyl-10-phenyl-4, 6-decandienoic acid (Adda). Structural variation can occur at all 7 positions, with

positions 2 and 4 (X and Z on Figure 1A, respectively), as the most variable positions. In addition, lack of methylation of Asp^3 and Dha^7 is a common feature (for review, see Welker and von Döhren, 2006). The microcystin isoforms are named according to the two most variable positions by applying the one-letter code for amino acids, e.g. microcystin-LR for the variant with Leu at position 2 (X) and Arg in position 4 (Z). At least 89 structurally different microcystin isoforms produced by diverse cyanobacterial genera like *Microcystis*, *Anabaena* and *Planktothrix* have been identified (Sivonen and Jones, 1999; Welker and von Döhren, 2006). Microcystin production is also known from a small number of planktonic, benthic and terrestrial strains of the genera *Nostoc* (Sivonen *et al*., 1992; Oksanen *et al*., 2004), *Hapalosiphon* (Prinsep *et al*., 1992) and *Phormidium* (Izaguirre *et al*., 2007). Nodularins, a family of hepatotoxic microcystin-like pentapeptides, are produced by *Nodularia* species (e.g. Sivonen *et al*., 1989; Jones *et al*., 1994). Microcystins and nodularins are inhibitors of protein phosphatases 1 and 2A (Honkanen *et al*., 1990; MacKintosh *et al*., 1990; Yoshizawa *et al*., 1990; Honkanen *et al*., 1991).

Cyanopeptolins (Figure 1B) are cyclic peptides that contain a unique 3-amino-6-hydroxy-2-piperidone (Ahp) residue and form a large group with high structural variability (Welker and von Döhren 2006). These peptides are produced by the freshwater genera *Anabaena* (Fujii *et al*., 1996; Fujii *et al*., 2002), *Microcystis* (e.g. Harada *et al*., 1993; Martin *et al*., 1993; Harada *et al*., 2001), *Nostoc* (e. g. Kaya *et al.*, 1996; Okino *et al*., 1997) and *Planktothrix* (Shin *et al*., 1995; Fujii *et al*., 2000; Blom *et al*., 2003); similar peptides have been isolated from terrestrial and marine cyanobacteria (e.g. Harrigan *et al*., 1999; Matern *et al*., 2001; Nogle *et al*., 2001; Matern *et al*., 2003). Many cyanopeptolins are serine protease inhibitors and several studies indicate that the Ahp residue plays an important role in the inhibition (Lee *et al*., 1994; Matern *et al*., 2003).

Aeruginosins (Figure 1C) are linear peptides characterized by the presence of a derivate of hydroxyphenyl-lactic acid (Hpla) at the N-terminus and containing the unusual amino acid, 2-carboxy-6-hydroxyoctahydroindole (Choi) (Murakami *et al*., 1995). These serine protease inhibitors are produced by *Microcystis* (e.g. Murakami *et al*., 1995; Matsuda *et al*., 1996; Ishida *et al*., 1999) and *Planktothrix* (Shin *et al*., 1997) and at present, 27 variants have been published (Welker and von Döhren, 2006). Variants with methyl-proline (mPro) instead of Choi have been isolated from *Nodularia* (spumigin, (Fujii *et al*., 1997).

Microginins (Figure 1D) are 4-6 amino acids long linear peptides containing a decanoic acid derivate, 3-amino-2-hydroxy-decanoic acid (Ahda) (Okino *et al*., 1993).

These peptides have been found only in *Microcystis* (e.g. Okino *et al*., 1993; Ishida *et al*., 2000) and *Planktothrix* (Sano and Kaya, 1997), but similar peptides containing different decanoic acid derivates have been isolated from *Nostoc* (Ploutno and Carmeli, 2002) and *Lyngbya* (Hooper *et al*., 1998).

Anabaenopeptins (Figure 1E), containing D-lysine and the unique ureido linkage, are produced by various cyanobacterial species like *Anabaena* (Harada *et al*., 1995; Fujii *et al*., 1996; Fujii *et al*., 2000), *Planktothrix* (Erhard *et al.*, 1999; Sano *et al.*, 2001), *Microcystis* (Williams *et al.*, 1996), *Nodularia* (Fujii *et al*., 1997) and *Aphanizomenon* (Murakami *et al*., 2000), but also by marine sponges (Kobayashi *et al*., 1991). Some of the peptides have been reported to be protease inhibitors (e.g. Itou *et al.*, 1999; Murakami *et al.*, 2000).

Microviridins (Figure 1F), cyclic peptides with the special structural feature of a tricyclic ring system, are the largest known cyanobacterial oligopeptides (Ishitsuka *et al*., 1990). Microviridins are produced by *Microcystis* (Ishitsuka *et al*., 1990; Okino *et al*., 1995; Rohrlack *et al*., 2003), *Planktothrix* (Shin *et al*., 1996; Fujii *et al*., 2000) and *Nostoc* (Murakami *et al.*, 1997).

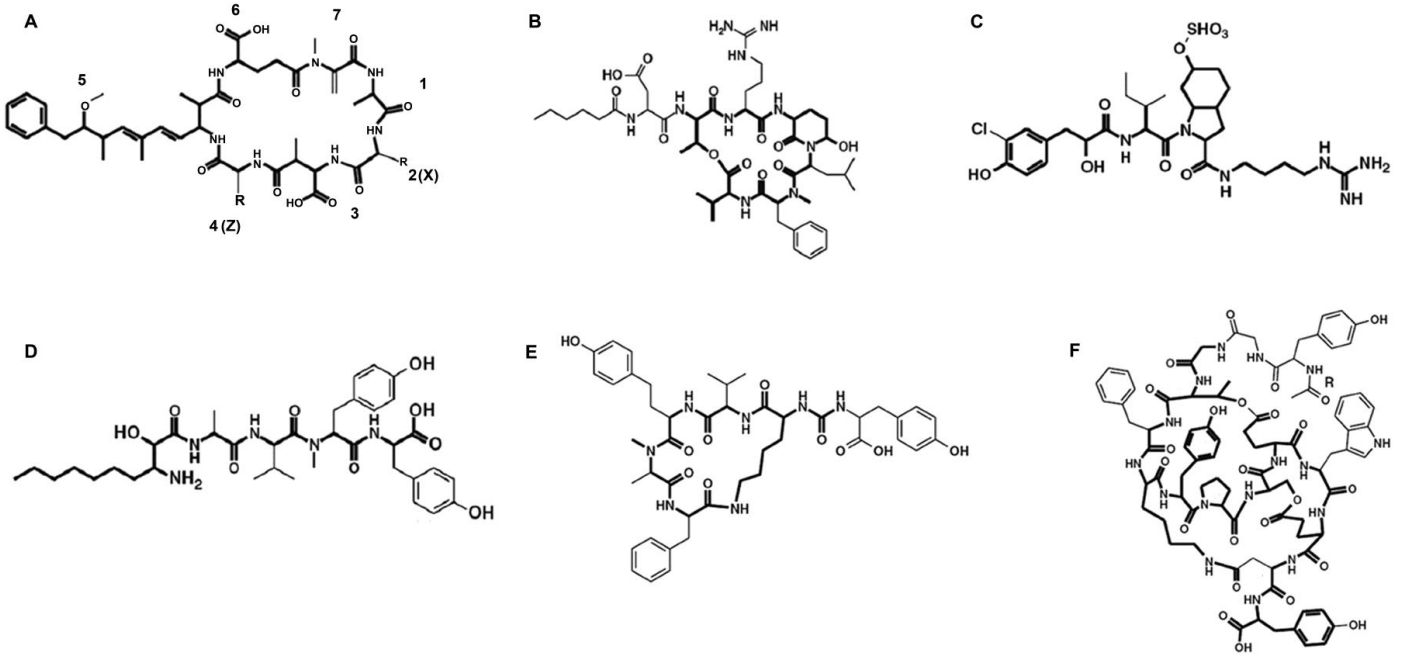

Figure 1. Examples of cyanobacterial peptides: (A) general structure of microcystin (Tillett *et al.*, 2000), the variable L-amino acid residues at positions 2 and 4 are indicated by X and Z, respectively. (B) cyanopeptolin A (Martin *et al.*, 1993), (C) aeruginosin 98-A (Murakami *et al.*, 1995), (D) microginin (Okino *et al.*, 1993), (E) anabaenopeptid A (Harada *et al.*, 1995), (F) microviridin A (Ishitsuka *et al.*, 1990).

1.3 Potential functions of cyanobacterial peptides

At present, only a few experimental studies have been published to resolve the ecological role of cyanobacterial oligopeptides and their biological role is poorly understood. However, several hypotheses on the function of peptides in the physiology and ecology of cyanobacteria have been discussed, mostly related either to grazing protection or to allelopathy.

The bioactivities exhibited by many cyanobacterial peptides towards mammalian (or vertebrate) test systems are often similar to effects observed in invertebrate animals that might be potential consumers of cyanobacteria. For a number of cyanopeptolins, aeruginosins and microviridins, a protease inhibitory activity has been reported (Shin *et al*., 1995; Shin *et al*., 1997; Ishida *et al*., 1999). The toxicity of microcystins to mammals is caused by the inhibition of protein phosphatases 1 and 2a, which are important enzymes in intracellular regulatory mechanisms (Honkanen *et al*., 1990; Dawson, 1998). A similar inhibition has been demonstrated for protein phosphatases of *Daphnia*, the most important grazer in pelagic freshwater systems (DeMott and Dhawale, 1995). In due course, it has been shown that an intoxication of *Daphnia* upon ingestion of cyanobacterial cells largely is dependent on the microcystin content of the cells (Rohrlack *et al*., 1999). However, no clear evidence indicates that *Daphnia* intoxication plays a major role in plankton dynamics. A recent phylogenetic analysis of the genes encoding the microcystin synthetase suggested that the ability to produce microcystins predates the metazoan lineage (Rantala *et al*., 2004) making it unlikely that microcystins evolved as a means of defense against grazers. Studies of several other NRPS gene clusters are needed to show whether this hypothesis can be extended to other nonribosomally produced peptides.

Allelopathy means that one organism harms another with specific biomolecules and allelopathic effects of cyanobacterial metabolites mostly concern the reduction of photosynthetic activity and growth rates of other planktonic autotrophs (Smith and Doan, 1999), eventually leading to cyanobacterial dominance (von Elert and Jüttner, 1997; Schagerl *et al*., 2002; Suikkanen *et al*., 2004). A recent review (Babica *et al*., 2006) showed that only a limited number of studies report harmful effects of microcystins at concentrations that are typical for the environment. Consequently, the ability of microcystins to act as general allelopathic compounds against photoautothrops seems unlikely.

Recently, the role as an infochemical that signals the extent of cell lysis in intraspecies communication has been proposed for nonribosomal peptides (Schatz *et al*., 2007). The presence of extracellular microcystin, microginin and micropeptin enhance the production of McyB and stimulate microcystin production in remaining intact *Microcystis* cells. In cases where cell death is driven by the action of a competitor, this response may enhance the defense capability of *Microcystis* (Schatz *et al.*, 2007). Microcystins have also been suggested to act as chelators in iron scavenging (Utkilen and Gjølme, 1995), as an internal nitrogen storage during N deficiency (Kotak *et al.*, 2000), in light adaptation processes (Hesse *et al.*, 2001), in inhibition of the carbon-fixating enzyme RuBisCo (Jähnichen *et al.*, 2001), and as mediators in colony formation (Kehr *et al*., 2006).

1.4 Nonribosomal peptide synthetases

The presence of nonproteinogenic amino acids and several studies indicate that the majority of cyanobacterial peptides are synthesized by nonribosomal peptide synthetases (NRPSs) or NRPS/polyketide synthase (PKS) hybrid pathways using the thiotemplate mechanism (Marahiel *et al.*, 1997).

1.4.1 Nonribosomal peptide synthesis versus ribosomal peptide/protein synthesis

Single elongation steps in ribosomal and nonribosomal peptide synthesis, respectively, are shown in Figure 2. The activation of the amino acid substrate is similar in both biosynthetic systems, but the enzymes involved, aa-tRNA synthetase in ribosomal and adenylation domains in nonribosomal synthesis, are structurally and catalytically unrelated (Pavela-Vrancic *et al*., 1994; Conti *et al*., 1997; Stachelhaus *et al*., 1998). The activated amino acid is loaded to the transport unit, tRNA in ribosomal and the thiolation domain (peptidyl carrier protein, PCP) in nonribosomal synthesis (Weber and Marahiel, 2001). In ribosomal peptide synthesis, peptide bond formation is directed by ribosomes and mRNA acts as a template determining the amino acid sequence of the product. The condensation domain catalyzes the formation of the peptide bond in nonribosomal peptide synthesis (Keating *et al.*, 2000).

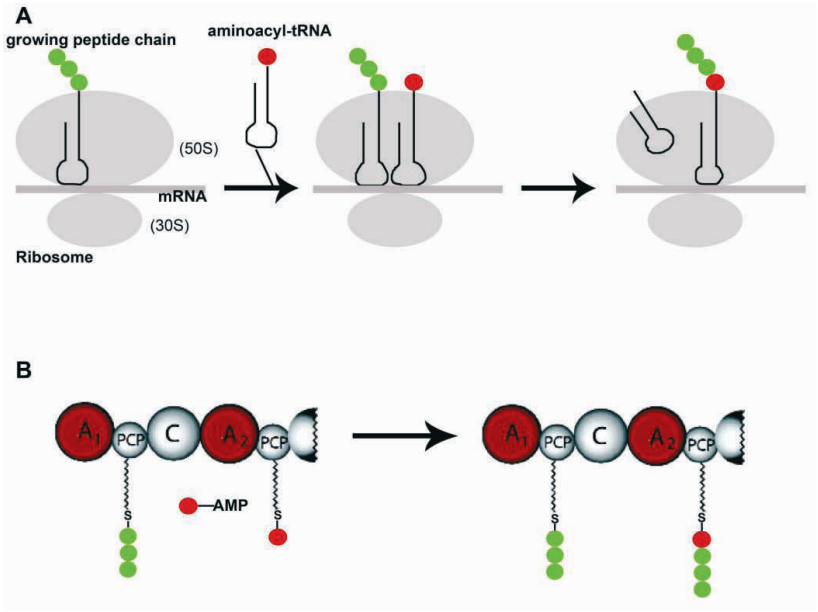

Figure 2. Comparison of ribosomal (A) and nonribosomal (B) peptide synthesis. A single elongation step of both types is shown. Green circles represent amino acids in the growing peptide and the red circle represents a new amino acid.

Both the ribosomal and nonribosomal machinery have properties that make them well-suited for their respective functions. Because of the need for precision in primary metabolism, ribosomal peptide synthesis involves several proofreading mechanisms that are absent from the nonribosomal system. However, ribosomal peptide synthesis is normally restricted to a set of 20 amino acids as building blocks for proteins, whereas several hundred substrates of NRPSs are known to date (Marahiel *et al.*, 1997). Thus, structural diversity is a predominant feature of nonribosomally produced peptides.

1.4.2 Modular structure of nonribosomal peptide synthetases

In ribosomal and nonribosomal peptide synthesis, the activation of amino acids is followed by peptide bond formation, and the steps are repeated until the final length of the peptide is attained. In NRPSs this is achieved through a modular structure, with one module for each amino acid to be incorporated into the peptide (Marahiel *et al.,* 1997; von Döhren *et al.*, 1997). The minimal module required for the addition of an amino acid to the growing peptide (Figure 3) consists of a condensation (C), an adenylation (A) and a thiolation (T) domain, (Marahiel *et al*., 1997; von Döhren *et al*., 1997; Stachelhaus *et al*., 1998).

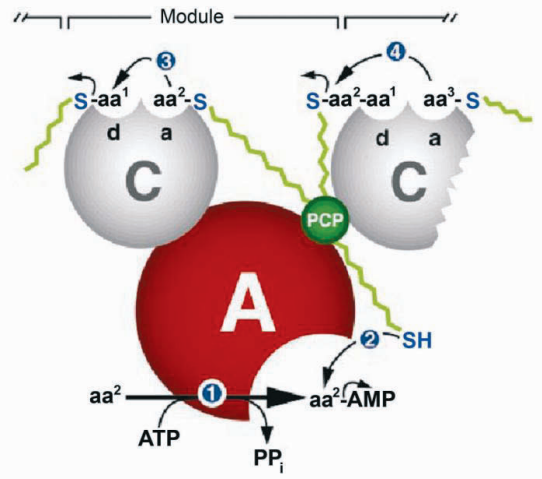

Figure 3. Overview of the composition and workflow of a module (Weber and Marahiel, 2001). The A-domain, indicated as a red ball, is involved in the selection and activation of the amino acid substrate (1), which then becomes covalently attached to the enzyme via a thioester bond to the phosphopantetheine group linked to the T-domain, indicated by a green ball, PCP (2). The C-domains (indicated by grey balls) catalyze the formation of the peptide bond between two aminoacyl moieties (3, 4). Donor and acceptor sites of the C-domains are indicated by d and a, respectively.

The A-domain recognizes the amino acid substrate and activates it, first through the formation of an aminoacyl adenylate and then via covalent binding of the activated amino acid as a thioester to the phosphopantetheine group of the T-domain. The C-domain catalyses the formation of a peptide bond between the aminoacyl or peptidyl moiety and the free amino group of the downstream aminoacyl moiety. In addition to these three core domains, modules can contain several alternative domains that introduce a modification of the amino acid being incorporated, such as a change of the Cα stereochemistry (epimerization domain), *N*-methylation (*N*-methyltransferase domain) of the α-amino group, or the heterocyclization of Ser, Thr or Cys residues (heterocyclization domain). The last step in nonribosomal peptide biosynthesis involves release of the assembled peptide from the NRPS into solution. In the majority of cases, this is accomplished by an extra domain located at the C-terminus of the last module of the NRPS, which can be a thioesterase (TE) domain, a variant of a C domain or a reductase domain. *In silico* recognition of individual domains on the protein level can be achieved using domain-specific highly conserved sequence motifs (Marahiel *et al.*, 1997).

In many NRPS gene clusters the order and number of modules is co-linear with the amino acid sequence and length of the peptide (Marahiel *et al*., 1997; von Döhren *et al*., 1997; Mootz *et al*., 2002). Consequently, it is possible by analysing the sequence of NRPS genes to elucidate the composition of the peptide, provided that the substrate specificities of the adenylation domains are known or can be deduced.

1.4.3 Substrate recognition by nonribosomal peptide synthetases

Several studies over the last decade have shown that individual domains within NRPSs exhibit significant substrate selectivity. A-domains which activate and transfer amino acids to T-domains (Figure 3) are considered to be the primary determinant of substrate selectivity. This is achieved by the geometry of a binding pocket in the enzyme that only allows specific amino acid(s) to enter into the catalytic site. The crystal structure of the Phe-activating A-domain of the gramicidin S-synthetase A allowed the identification of amino acid residues that play a decisive role in the coordination of the substrate and have lead to the concept of the so-called nonribosomal code, which allows the prediction of A-domain selectivity on the basis of its primary sequence (Conti *et al*., 1997; Stachelhaus *et al*., 1998; Challis *et al*., 2000; Lautru and Challis, 2004). This specificity conferring code has been confirmed in a variety of correlations on NRPS genes with known peptide products and has been used for prediction of unknown products (Challis and Ravel, 2000).

The studies of C-domain specificity have revealed that C-domains exhibit a strong stereoselectivity (L- or D-form of the amino acid) at their acceptor site (Figure 3) (Belshaw *et al*., 1999; Ehmann *et al*., 2000; Linne and Marahiel, 2000; Luo *et al*., 2002; Clugston *et al*., 2003; Samel *et al*., 2007). Some selectivity towards the side chain of the aminoacyl thioester at the acceptor site has also been shown (Ehmann *et al.*, 2000). Such selectivities imply the probable existence of a binding pocket of the amino acid at the acceptor site, and explain why elongation modules cannot initiate peptide chain synthesis and how directionality of nonribosomal peptide biosynthesis is controlled (Linne and Marahiel, 2000). Broader substrate specificity is observed at the donor (peptidyl) site (Figure 3) of C-domains, which has been shown to accept noncognate substrates, e.g. amino acyl instead of peptidyl moieties, or peptidyl chains with different size and amino acid composition (Belshaw *et al*., 1999; Doekel and Marahiel, 2000; Marshall *et al*., 2001; Linne *et al*., 2003). The stereochemistry of the C-terminal amino acid of the peptidyl chain appears, however, to be an important element in substrate recognition, as observed for the acceptor site (Ehmann *et al.*, 2000; Luo *et al.*, 2002).

1.5 Cyanobacterial nonribosomal peptide synthetases with characterized gene clusters

Genes encoding the NRPSs are usually organized in operons and in recent years, a number of NRPS/PKS gene clusters and operons have been characterized in cyanobacteria.

1.5.1 Microcystin (and nodularin) synthetases

Microcystins (Figure 1A) are produced by a microcystin synthetase complex, composed of several peptide synthetases and polyketide synthases (Figure 4). Microcystin synthetase is encoded by the *mcy* gene cluster characterized from all main microcystin producing genera: *Microcystis* (Nishizawa *et al.*, 1999; Nishizawa *et al.*, 2000; Tillett *et al.*, 2000), *Planktothrix* (Christiansen *et al.*, 2003) and *Anabaena* (Rouhiainen *et al.*, 2004). The structural organization of the biosynthetic NRPS/PKS genes, including their modular arrangement, has been conserved in all three genera. Differences in the clusters have been found with respect to the arrangement of genes, the location and orientation of promoter regions and the content of genes not directly involved in the peptide assembly (Figure 6, chapter 1.6).

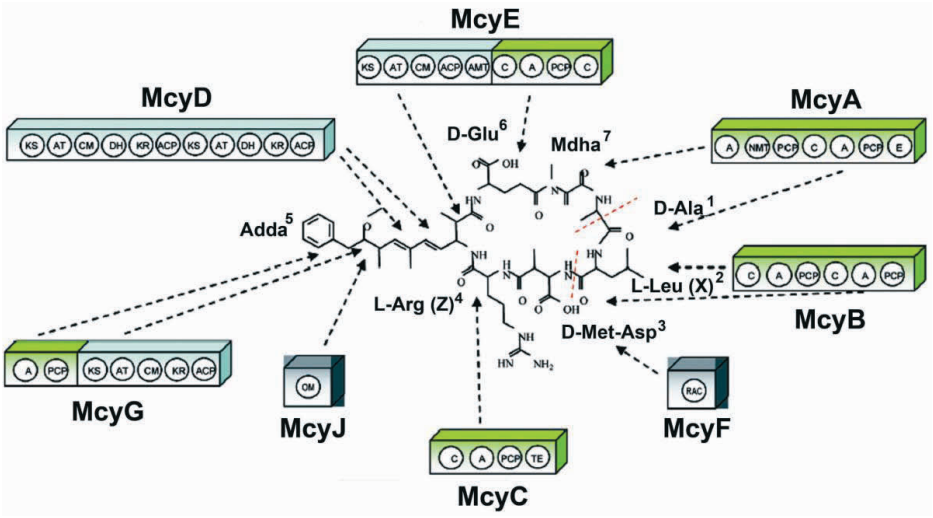

Figure 4. Model of the microcystin synthetase (producing microcystin-LR) domain structures (from Dittmann and Wiegand, 2006). A: adenylation domain, C: condensation domain, PCP: thiolation domain, KS: b-ketoacyl synthase, AT: acyltransferase, ACP: acyl carrier protein, KR: ketoacyl reductase, DH: dehydratase, CM: C-methyltransferase, OM: O-methyltransferase, NMT: *N*-methyltransferase, AMT: aminotransferase, RC, racemase. Polyketide synthase (PKS) domains are indicated in blue, nonribosomal peptide synthetases (NRPS) domains are indicated in green, and tailoring proteins are indicated in grey boxes. Red lines are bordering the two amino acid moieties lacking in the nodularin structure.

McyA, McyB and McyC are three NRPSs containing five of the seven modules needed for microcystin biosynthesis. McyE and G are hybrid enzymes including both peptide synthetase and polyketide synthase modules while McyD is a two-modular

polyketide synthetase. The two PKS modules of McyG and McyE, together with McyD, are responsible for the synthesis of the unique Adda moiety of microcystins (Nishizawa *et al.*, 2000; Tillett *et al.*, 2000). The amino acids at the variable positions 2 and 4 are incorporated by the first module of McyB and by McyC, respectively. An ABC transporter (McyH), found in all sequenced *mcy* gene clusters, has been shown to be essential for microcystin production (Pearson *et al.*, 2004). Another protein found in all genera is McyJ, an O-methyltransferase (Nishizawa *et al*., 1999; Nishizawa *et al*., 2000; Tillett *et al*., 2000; Christiansen *et al*., 2003; Rouhiainen *et al*., 2004). Several studies have shown that a single enzyme system in each strain produces a set of microcystin isoforms (Dittmann *et al*., 1997; Nishizawa *et al*., 1999; Nishizawa *et al*., 2000; Christiansen *et al*., 2003).

A comparison of microcystin and nodularin biosynthetic genes supports the close relationship between these systems and suggests that the nodularin synthetase gene cluster evolved from the microcystin cluster by domain deletion (Moffitt and Neilan, 2004; Rantala *et al*., 2004). Indeed, the two amino acids following the dehydro-residue in position 3 in microcystins are missing in nodularin (Figure 4). The respective modules corresponding to parts of McyA and McyB are missing, and the remains are fused into the two-module synthetase NdaA. All other genes have orthologues in the microcystin cluster (Figure 6).

1.5.2 Anabaenopeptilide (cyanopeptolin) synthetase

Anabaenopeptilides (Fujii *et al.*, 1996) are members of the cyanopeptolin class of cyanobacterial peptides (Figure 1B). The anabaenopeptilide synthetase gene cluster from *Anabaena* contains three genes (*apdA*, *B* and *D*) coding for NRPSs, with seven modules in total, and a gene (*apdC*) encoding a putative halogenase expected to be involved in chlorination of a Tyr residue (Figure 5) (Rouhiainen *et al.*, 2000). A putative formyl-transferase domain, expected to formylate the side chain amino acid residue, is located at the N-terminus of ApdA and was first described in this system. The unusual amino acid Ahp is proposed to be generated from Gln/Glu and the adjacent Thr by reduction of the enzyme bound glutamate to glutamic semialdehyde (catalyzed by a putative acyl carrier protein reductase, ApdF), which then, by condensation is linked to the nitrogen of the next residue (catalyzed by methyltransferase domain of module 5; Rouhiainen *et al.*, 2000).

The ABC transporter encoding gene is found downstream of the *apd* operon.

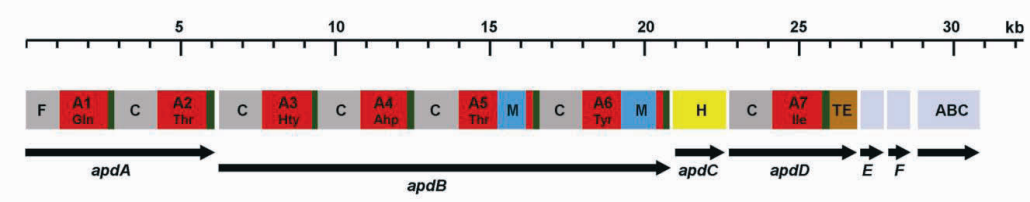

Figure 5. Organization and modular structure of anabaenopeptilide synthetase genes (modified from Rouhiainen *et al.*, 2000). The domains encoded by the genes are indicated as follows: C – condensation domain, grey box; A – adenylation domain (the predicted amino acid that is activated is noted), red box;
M – *N*-methyltransferase, blue box; H – halogenase, yellow box; TE – thioesterase, brown box; T – thiolation domain (PCP), green box. The ABC transporter (ABC) is indicated by a light blue box.

1.5.3 Aeruginoside synthetase

Aeruginosides (Figure 1F) are produced by several freshwater cyanobacterial genera (for a review, see Welker and von Döhren, 2006). Recently, a gene cluster encoding aeruginoside synthetase was characterized from *Planktothrix* (Ishida *et al.*, 2007). The aeruginoside biosynthetic cluster contains one PKS gene (*aerA*), two NRPS genes (*aerB* and *aerG*) and several genes encoding tailoring enzymes. Proteins AerC, AerD, AerE and AerF are proposed to provide the unique Choi moiety (Ishida *et al.*, 2007).

1.5.4 Nonribosomal peptide synthetases from marine cyanobacterium *Lyngbya majuscula*

Strains of *Lyngbya majuscula* produce many bioactive metabolites (Gerwick *et al*., 1992; Burja *et al*., 2001) and three NRPS/PKS encoding gene clusters have been characterized from this species. The chlorinated lipopeptide barbamide is synthesized by a mixed NRPS/PKS system. Several unique features like trichloro-Leu as a starter unit, unique biochemical mechanisms of chlorination, E-double bond formation and thiazole ring formation are included in the biosynthesis of barbamide (Chang *et al.*, 2002). Curvasin A, a polyketide with a single cysteine converted to thiazolidine, is a potent cancer cell toxin (Wipf *et al.*, 2004). Curvasin is synthesized by what mainly is a PKS system with only one NRPS module (Chang *et al.*, 2002). The tailoring enzyme HMG-CoA synthase is likely to be responsible for formation of the cyclopropyl ring. A biosynthetic gene cluster has also been characterized for another *Lyngbya* polyketide, jamaicamide (Edwards *et al.*, 2004). The lyngbyatoxins are potent skin irritants with a prenylated indolactam structure derived from Val and Trp (Edwards and Gerwick, 2004). The biosynthetic gene cluster encodes a

two-module NRPS, a P450 mono-oxygenase, an aromatic prenyltransferase and an oxidase/reductase protein (Edwards and Gerwick, 2004).

1.5.5 Nonribosomal peptide synthetases from terrestrial *Nostoc* strains

Two nonribosomal peptide biosynthetic clusters have been characterized from terrestrial *Nostoc* strains: nostopeptolide synthetase (Hoffmann *et al.*, 2003) and nostocyclopeptide synthetase (Becker *et al.*, 2004). Nostopeptolides are branched acylated octapeptides with a heptapeptide lactone structure (Golakoti *et al.*, 2000). The *nos* gene cluster encodes a mixed NRPS/PKS system. A zinc-dependent long-chain dehydrogenase and a δ(1)-pyrroline-5-carboxylic acid reductase are involved in the formation of mPro (Luesch *et al.*, 2003). A gene encoding an ABC transporter is included to *nos* gene cluster (Hoffmann *et al.*, 2003). Nostocyclopeptides are cycloheptapeptides containing methyl-Pro similarly to nostopeptolides. The *ncp* gene cluster contains NRPS encoding genes for assembly of the peptide and additional genes, orthologues to the respective *nos*-genes involved in mPro supply. A gene encoding an ABC-transporter is found in the *ncp* gene cluster and a putative transposase is located downstream of *ncpA* (Becker *et al.*, 2004).

1.6 Evolution of nonribosomal peptide synthetases

Our understanding of the evolution of NRPS systems is rather poor. Recently, progress has been made in the elucidation of the genetic basis of microcystin synthesis for all three main microcystin producing genera, i.e., *Anabaena*, *Microcystis* and *Planktothrix* (Nishizawa *et al*., 1999; Nishizawa *et al*., 2000; Tillett *et al*., 2000; Christiansen *et al*., 2003; Rouhiainen *et al*., 2004). The characterization of the *mcy* gene clusters from these genera has made it possible to draw conclusions about the evolution of microcystin synthetase genes. The nodularin synthetase gene cluster (*nda*) from *Nodularia* (Moffitt and Neilan, 2004) has been included to this overview due to close relation to the *mcy* gene cluster.

1.6.1 Comparison of *mcy* gene clusters between distantly related cyanobacteria

Although the multienzyme components encoded by *mcy* gene clusters from different genera are highly similar, the principle organization of the gene clusters is different in the individual genera (Figure 6). In *Microcystis*, *Anabaena*, and *Nodularia*, the genes are

transcribed from a central bidirectional promoter region, whereas in *Planktothrix* all *mcy* genes except *mcyT* seem to be transcribed unidirectionally from a promoter located upstream of *mcyD* (Figure 6). The arrangement of their genes clearly differs between *Anabaena* and *Nodularia* on one side and *Microcystis* and *Planktothrix* on the other. Similarly, the positions of tailoring genes and the gene encoding a putative ABC transporter differ between the genera, except in *Anabaena* and *Nodularia*, where remarkably the only difference is the lack of two modules and fusion of *mcyA* and *mcyB*, as seen from an analysis of the *ndaA* gene of the nodularin biosynthesis gene cluster (Moffitt and Neilan, 2004). These striking organizational differences indicate individual evolution of the *mcy* gene clusters within *Anabaena*, *Microcystis* and *Planktothrix* and do not support the idea of frequent horizontal transfer between the genera.

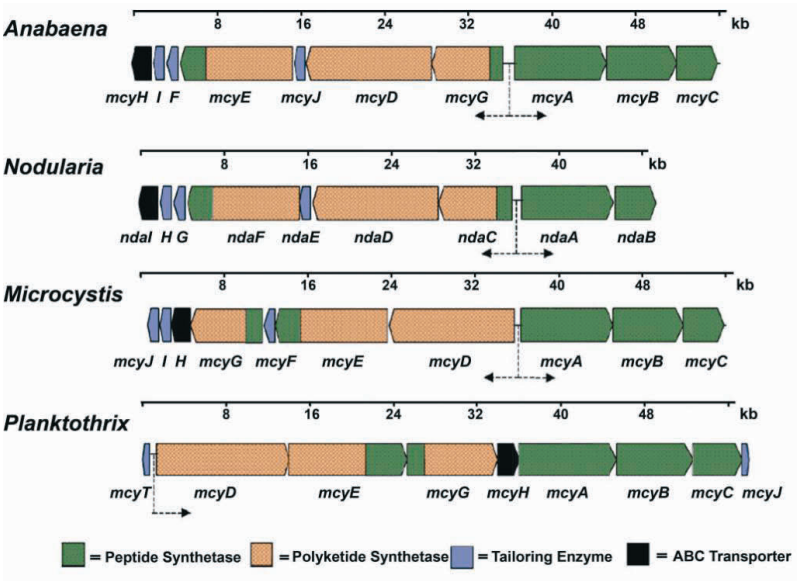

Figure 6. Gene clusters coding for the biosynthesis of microcystin in *Anabaena* (Rouhiainen *et al.*, 2004), *Microcystis* (Tillett *et al.*, 2000), *Planktothrix* (Christiansen *et al.*, 2003), and of nodularin in *Nodularia* (Moffitt and Neilan, 2004). Arrows indicate the transcriptional start sites from the putative promoter regions. Modified from Dittmann and Börner (2005).

Rantala and co-workers (2004) have analyzed *mcyA*, *mcyD* and *mcyE* sequences from distantly related strains (from different genera) and compared the *mcy* phylogeny with the phylogeny of housekeeping genes (16S rRNA and *rpoC1*). The high degree of congruence found between these phylogenies indicates that the housekeeping genes and *mcy* genes have evolved for the entire evolutionary history of microcystin and that *mcy* genes

were present in the last common ancestor of a large number of cyanobacteria. The patchy distribution of *mcy* genes within individual genera was explained by repeated loss of the *mcy* gene cluster during cyanobacterial evolution (Rantala *et al.*, 2004). Reports about strains possessing the *mcy* genes but lacking microcystin production (Tillett *et al*., 2001; Mikalsen *et al*., 2003; Kurmayer *et al*., 2004; Mbedi *et al*., 2005; Christiansen *et al*., 2006) support the idea of a frequent loss of the ability to synthesize microcystin during evolution. No clear evidence of horizontal transfer of *mcy* genes between *Anabaena*, *Microcystis* and *Planktothrix* strains has been shown to date, suggesting independent evolution of *mcy* genes within each genus. Unlike the *mcy* genes, sequence information on the genes involved in the synthesis of other secondary metabolites is limited and a phylogenetic study is not available. Such studies may indicate whether the hypothesis of a common ancestor and independent evolution within different cyanobacterial genera can be extended to other NRPS gene clusters.

1.6.2 Processes reshaping *mcy* genes within closely related strains

Several studies have shown that the rate of successful homology-driven recombination rapidly is reduced with increased genetic distance (Rudi *et al*., 1998; Zhaxybayeva *et al*., 2006; Papke *et al*., 2007), explaining the lack of examples of horizontal transfer of *mcy* genes between different genera. Recombination within a genus would be is expected to occur much more frequently. Indeed, several recombination events in *mcy* genes have been shown within *Microcystis* and *Planktothrix*. Tanabe and co-workers (2004) reported recombination within *mcyA*, but not within *mcyD*, *mcyG* and *mcyJ* genes of closely related *Microcystis* strains. However, incongruence between the four *mcy* gene phylogenies indicated that recombination has occurred over the entire *mcy* gene cluster. Kurmayer and Gumpenberger (2006) reported that parts of the *mcyB* gene frequently have been transferred between lineages of *Planktothrix* spp. leading to a mosaic gene structure rather than a bifurcating phylogenetic tree. The analysis of a panel of *mcy* genes from related *Anabaena* strains most likely would detect recombination also within this genus.

Two reported recombination events have lead to replacement of whole domains and production of different microcystin isoforms. Mikalsen and co-workers (2003) showed that a recombination event between two different adenylation (A)-domain encoding segments has lead to presence of the two different types of A-domain in the first module of McyB (McyB1). Strains containing the A-domain similar to corresponding domain of McyC in

module McyB1 (McyB1 C-like) produce mainly microcystin-RR, while strains containing the second type of A-domain in McyB1 (McyB1 B-like) produce mainly microcystin-LR. A single recombination breakpoint was identified in the McyB1 A-domain of *Microcystis*, but the precise pattern of this replacement was not elucidated (Mikalsen *et al*., 2003). Similarly, in some *Planktothrix* strains, the typical Ser-activating and *N*-methyltransferase (NMT) containing A-domain in the first module of McyA (McyA1) has been replaced by a Thr-activating A-domain without NMT. This recombination event resulted in the production of the unmethylated Dhb^7-microcystin isoforms instead of $Mdha^7$-isoforms (Kurmayer *et al.*, 2005). The flanking regions as well as the site of the recombination in A-domain of McyA1 were found to be identical within all 12 *Planktothrix* strains containing the replaced domain, implying one single recombination event (Kurmayer and Gumpenberger, 2006).

An excess of synonymous over non-synonymous substitutions (ω–values below 1) as shown by several studies, (Rantala *et al*., 2004; Tanabe *et al*., 2004; Kurmayer and Gumpenberger, 2006) indicates that *mcy* genes are subject to purifying selection and that mutations affecting the amino acid sequence in general are deleterious. However, averaging ω-values over all sites of protein typically underestimates positive selection because the ratio is overwhelmed by the ubiquitous purifying selection. Kurmayer and Gumpenberger (2006) have tested for positive selection acting on specific sites of specific genotypes of *mcyB* and reported that some sites have experienced a relaxation of selective constraints. Further analysis using site-specific models might reveal the presence of sites under positive selection within different domains of microcystin synthetase.

2. AIMS OF THE STUDY

Despite the large number of cyclic peptides found in cyanobacteria, the only nonribosomal peptide synthetase gene cluster that has been characterized in several genera is the microcystin synthetase gene cluster, and our understanding of the evolution of nonribosomal peptide synthetases is rather poor.

The overall aim of this study was to gain further insight into the evolution of genes encoding peptide synthetases in cyanobacteria. One of the main objectives was to evaluate the importance of various genomic processes shaping the microcystin synthetase gene clusters and thus the structure of microcystin isoforms. One of our aims was to investigate the evolution of another cyanobacterial nonribosomal peptide synthetase. Since a cyanopeptolin synthetase gene cluster from *Anabaena* has been characterized earlier, this part of the work has mainly aimed at studying the cyanopeptolin synthetase gene clusters in other cyanobacterial genera and comparison of these to deduce possible co-evolution and cross-NRPS recombination.

This included studies of;

1. microcystin synthetase *mcyABC* genes within three main microcystin-producing genera; *Anabaena*, *Microcystis* and *Planktothrix*.
2. cyanopeptolin gene clusters in *Microcystis* and *Planktothrix*, characterization and comparison with equivalent gene cluster from *Anabaena*. Comparison with microcystin synthetase gene clusters from same three genera.

3. SUMMARY OF PAPERS

3.1 Paper I

The *mcyABC* operon is a genetic mosaic in naturally occurring *Microcystis* strains (Manuscript submitted to Environmental Microbiology).

Tooming-Klunderud, A., Mikalsen, B., Kristensen, T. and K.S. Jakobsen

An extensive study of the *mcyABC* operon and the flanking regions of the *mcy* gene cluster was performed in a number of naturally occurring *Microcystis* strains. Several of the strains examined produce only desmethyl[7]-microcystin isoforms, thus the segment of *mcyA* encoding the *N*-methyltransferase (NMT) domain of the first module of McyA (McyA1) was investigated. Both Southern hybridization and PCR results indicated the presence of a NMT domain in all toxic strains, and the lack of methyltransferase activity was found to be associated with specific point mutations. Alignments of the various regions, splits trees analyses and statistical tests all showed that multiple recombination events have occurred in the *N*-methyltransferase domain encoded by *mcyA* and the adenylation domains encoded by *mcyB* and *mcyC* giving, rise to "phylogenetic mosaics". In addition, recombinations were detected between the *mcyB* segment encoding the arginine-activating adenylation domain of the first module of McyB and the *mcyC* adenylation domain region. The precise pattern of a previously reported replacement of the adenylation domain in the first module of McyB was found to involve the region between the conserved motifs A3 and A8/A9. All microcystin-producing *Microcystis* strains contained the same flanking genes (*dnaN* and *uma1*). Clear indications of recombination, an insertion element and footprints of several IS-elements were found in intergenic region between *dnaN* and *mcy* operon. Among the non-microcystin-producing strains, the close linkage between *dnaN* and *uma1* was not conserved, since in two strains a gene with similarity to *ftsH* encoding an ATP-dependent Zn protease, was found instead of *dnaN*. The majority of the non-microcystin producing strains completely lacked *mcy* genes, while one strain possessed a partially deleted *mcy* operon. Taken together, the results show that the *mcy* gene cluster variation is caused by frequent horizontal gene transfer events and most likely intragenomic recombinations involving IS-elements, in addition to point mutations and insertions/deletions.

3.2 Paper II

Microcystin synthetase *mcyABC* operon adenylation domains from three cyanobacterial genera: domain –and group-specific differences in recombination rates and selectional forces (manuscript).

Tooming-Klunderud, A., Fewer, D., Rohrlack, T., Jokela, J., Rouhiainen, L., Sivonen, K., Kristensen, T. and K.S. Jakobsen

In this study, a comparative analysis of adenylation domains in the first module of McyB and McyC in the microcystin synthetase gene cluster was performed on a large number of naturally occurring microcystin-producing strains from the cyanobacterial genera *Anabaena*, *Microcystis* and *Planktothrix*. We found no clear signs of recombination between genera, while frequent recombination events involving *mcyB* gene sequences were detected between strains within the same genus and *mcyC* sequences in *Anabaena* and *Microcystis*. Frequent recombination events were also observed between *mcyB* and *mcyC* sequences in *Anabaena* and *Microcystis*, but not in *Planktothrix*. Recombination/mutation rate ratios suggest that the diversification of *mcyB* and *mcyC* genes is caused by recombinations as well as point mutations in all three genera. A notable exception here is *Planktothrix mcyC* showing no detectable recombinations. While the adenylation domains of the first domain of McyB and McyC overall seem to be under purifying selection, some amino acid residues appear to be under a positive selective pressure. These include residues important for the active site selectivity of the A-domain, indicating selection for new microcystin variants.

3.3 Paper III

An in-frame deletion in the N-methyltransferase domain of the microcystin synthetase gene *mcyA* in strains of genus *Anabaena*
(Submitted).

Fewer, D., Tooming-Klunderud, A., Jokela, J., Rouhiainen, L., Kristensen, T., Rohrlack, T., Jakobsen, K.S. and K. Sivonen.

In this work, the N-methyltransferase (NMT) domain of the first module of McyA (McyA1) was investigated in a number of *Anabaena* strains. We report a novel 1236 bp in-frame deletion of the *mcyA* gene in 9 strains removing almost the entire NMT domain from *mcyA*. *Anabaena* strains carrying the in-frame deletion produced desmethyl-microcystin isoforms, incorporating mainly dehydroalanine (Dha). The remaining strains with full length *mcyA* genes incorporate mainly N-methyldehydroalanine (Mdha). Interestingly, the strains lacking the NMT domain also incorporate elevated amounts of L-Ser, the precursor of Dha and Mdha, into the microcystin they produce relative to strains carrying functional NMT domains. The production of desmethyl-microcystin isoforms was found even in the presence of a functional NMT domain. The simultaneous production of Mdha and Dha containing microcystins in *Anabaena* is most likely the result of domain skipping in McyA with the biosynthesis of microcystins proceeding without N-methylation taking place.

3.4 Paper IV

Structural analysis of a non-ribosomal halogenated cyclic peptide and its putative operon from *Microcystis*: implications for evolution of cyanopeptolins.

Tooming-Klunderud, A., Rohrlack, T., Shalchian-Tabrizi, K., Kristensen, T. and K.S. Jakobsen (2007). Microbiology 153, 1382–1393.

In this study, the putative cyanopeptolin gene cluster from *Microcystis* cf. *wesenbergii* NIVA-CYA 172/5 which produces only cyanopeptolin was characterized. The structure of the major peptide produced by this strain, the halogenated heptapeptide cyanopeptolin-984, was determined using LC/MS/MS. A 29 kb *mcn* gene cluster encoding a peptide synthetase putatively producing a cyanopeptolin was cloned and sequenced. The cluster consists of four genes encoding peptide synthetases, containing seven modules in total, and a gene encoding a halogenase. Two additional ORFs transcribed in the opposite direction were found in the flanking sequence; one of these encodes an ABC transporter. The overall organization of the cyanopeptolin synthetase operon (*mcn*) resembles a previously analyzed anabaenopeptilide synthetase operon (*apd*) from *Anabaena* strain 90. Phylogenetic analyses of the individual domains from Mcn, Apd and other cyanobacterial peptide synthetases showed clustering of the adenylation domains according to function irrespective of operon origin – indicating strong functional constraints across peptide synthetases. In contrast, the condensation and thiolation domains to a large extent grouped according to operon affiliation or position in the respective operons. Phylogenetic analyses of condensation domains indicated that N-terminal domains and domains that condense L-amino acids and D-amino acids, respectively, form three separate groups. Although recombination events are likely to be involved in the evolution of *mcn*, no clear evidence of genetic recombination between the two cyanopeptolin gene clusters was found. Within the genus *Microcystis*, microcystin and cyanopeptolin synthetases have an evolutionary history of genomic coexistence. However, the data indicated that the two classes of peptide synthetase gene clusters have evolved independently.

3.5 Paper V

Comparison of cyanopeptolin genes in *Planktothrix*, *Microcystis* and *Anabaena*: evidence for independent evolution within each genus.

Rounge, T.B., Rohrlack, T., Tooming-Klunderud, A., Kristensen, T. and K.S. Jakobsen. Applied and Environmental Microbiology, Vol. 73, No. 22, 7322-7330

A putative cyanopeptolin gene cluster was characterized from *Planktothrix* NIVA CYA 116, which only produces cyanopeptolins. The major cyclic peptide, cyanopeptolin-1138, produced by this strain was characterized and shown to be structurally very close to the earlier characterized oscillapeptin E. A cyanopeptolin gene cluster likely to encode the corresponding peptide synthetase, the 30 kb *oci* gene cluster, contains two novel domains that previously have not been detected in NRPS gene clusters: a putative glyceric acid activating domain and a sulfotransferase domain, in addition to seven NRPS modules. Unlike the two previously described cyanopeptolin gene clusters from *Anabaena* and *Microcystis,* no halogenase gene is present. Comparison of the three known cyanopeptolin gene clusters from distantly related genera shows similar gene and domain arrangements, while the binding pocket signature sequences, deduced from the A-domain sequences and the additional tailoring domains vary. This suggests independent loss and gain of tailoring domains within each genus as major events leading to the present diversity. Phylogenetic analysis of the ABC transporter genes associated with the NRPS gene clusters revealed the presence of a cyanopeptolin-specific clade, indicating that the ABC-transporter genes have evolved as part of the functional unit. Phylogenetic analyses of A- and C-domains, including domains from cyanopeptolins and microcystins, show a closer similarity between the *Planktothrix* and *Microcystis* cyanopeptolin-domains compared to *Anabaena*. There were no strong indications of horizontal gene transfer of cyanopeptolin gene sequences between the three genera, supporting independent evolution within each group of cyanobacteria. Comparison of microcystin and cyanopeptolin synthetase gene clusters from three genera revealed that although these gene clusters have co-existed in several strains; no clear evidence for recombination between gene clusters could be detected.

4. DISCUSSION

The discussion is divided in two parts; (1) evolution of the microcystin synthetase (*mcy*) gene cluster within the three main microcystin-producing cyanobacterial genera *Microcystis*, *Anabaena* and *Planktothrix,* and (2) comparison of cyanopeptolin synthetase gene clusters from same cyanobacterial genera.

4.1 Evolution of the microcystin synthetase gene cluster

4.1.1 Genomic processes and natural selection shaping microcystin-isoforms produced by *Anabaena*, *Microcystis* and *Planktothrix*

The microcystin synthetase domains investigated in Papers I-III contribute to microcystin-isoform diversity. The *N*-methyltransferase (NMT) domain in the module McyA1 catalyses the *N*-methylation of amino acid residue in position 7 and the adenylation (A)-domains in modules McyB1 and McyC activate amino acids that are incorporated in the variable positions X and Z of microcystin (Figure 4).

Methylation of the amino acid residue in position 7 in microcystin

In general, *N*-methylation of amino acid residues stabilizes the peptide bond against proteolytical cleavage and can also contribute to the biological activity of the peptide (Marahiel *et al.*, 1997). The *N*-methyl group in microcystin can have a pronounced effect on toxicity towards eukaryotes: the [Dha^7]-MC-LR isoform is 5 times less toxic than the MC-LR, while the [Dha^7]-MC-RR is 3 times more toxic than the MC-RR variant (Sivonen and Jones, 1999). The missing methyl-group could also affect the polarity of the microcystin and transport of the peptide into the cell.

Strains producing merely desmethyl^7-microcystin isoforms have been reported from *Anabaena*, *Microcystis* and *Planktothrix* (e.g. Sivonen *et al*., 1992; Nishizawa *et al*., 1999; Fastner *et al*., 2001; Mikalsen *et al*., 2003; Kurmayer *et al*., 2005) indicating that methylation of amino acid residue at position 7 is not crucial for the function of microcystins. The simultaneous production of Mdha^7- and Dha^7-containing microcystins has also been shown for all genera (Luukkainen *et al*., 1993; Robillot *et al*., 2000; Haande *et al*., 2007, Paper III) and is most likely the result of the NMT-domain being skipped during the assembly of Dha containing microcystins. In *Planktothrix* strains, the production of only

desmethyl^{7}-microcystin is correlated with lacking NMT-domain in module McyA1 (Kurmayer *et al.*, 2005). Our results show that absence of a NMT-domain (due to an in-frame deletion) also correlates with desmethyl^{7}-microcystin production in *Anabaena* strains (Paper III), while point mutations in functionally important sites seem to have inactivated the NMT-domain in desmethyl^{7}-microcystin producing *Microcystis* strains (Paper I).

Methylation of the amino acid residue in position 7 seems to be advantageous since most strains contain a NMT-domain in McyA1 and produce Mdha^{7}-isoforms. However, genotypes with missing/inactive NMT-domains are observed in all genera (Kurmayer *et al.*, 2005; Papers I and III), indicating that desmethyl^{7}-isoforms are useful for some strains.

Recombination is shaping *mcyABC* genes.

Evolution of secondary metabolic pathways is probably a result of modification and novel combination of reactions from preexisting pathways. The modular structure of nonribosomal peptide synthetases and presence of highly conserved regions within domains imply that also the gene clusters encoding the NRPS complexes are modular and have conserved regions in common. Because of these conserved sequence regions, such modular gene clusters should be well suited to participate in various homology-driven recombination events. Adenylation domains contain 10 highly conserved core motifs (Marahiel *et al.*, 1997) and DNA segments encoding these may serve as hotspots in recombination between A-domain encoding sequences. Recombination may occur between A-domain encoding sequences from different NRPS gene clusters and different genera if the donor and recipient DNA molecules show high enough level of sequence similarity (Shen and Huang, 1986; Majewski and Cohan, 1999; Lovett *et al*., 2002). Recombination events between different genera have been observed where donor and recipient sequences differ at as many as 25-30% of the nucleotide sites (for review, see Feil and Spratt (2001)). Comparison of *mcyB1* and *mcyC* sequences from *Anabaena*, *Microcystis* and *Planktothrix* showed 27-34 % and 18-29 % sequence variation within *mcyB1* and *mcyC*, respectively (Paper II), suggesting that homology-driven recombination between these genera might occur, but probably with very low frequency. Phylogenetic and recombination analyses revealed no signs of recombination between genera in our dataset of 108 A-domains from 58 cyanobacterial strains, indicating that the frequency of HGT involving *mcy* genes from *Anabaena*, *Microcystis* and *Planktothrix* must be low.

Recombination events leading to reshaping of A-domains of McyB1 and McyC may contribute to the production of specific/novel microcystin isoforms. This has been shown for *Microcystis* spp., where a recombination event between two A-domain encoding sequences has lead to presence of two different types of A-domains in McyB1 (Mikalsen *et al.*, 2003). Recombination between *mcyB1* and *mcyC* was suggested as the reason for the similarity between the A-domains in McyB1 and McyC in some strains. Another possibility is that the A-domains of McyC and McyB1 were similar in the ancestral gene cluster. If so, some *Microcystis* strains must have acquired a novel type of A-domain in McyB1 (called B-like by Mikalsen *et al.* (2003)). Since no A-domain similar to the B-like in McyB1 is present in microcystin synthetase, the B-like A-domain must have been introduced into Mcy from another NRPS system. Similarly, recombination between sequences coding for two different NRPS has resulted in replacement of the NMT-containing A-domain in McyA1 with another A-domain without a NMT in some *Planktothrix* strains (Kurmayer *et al.*, 2005).

Recombination events involving *mcy* genes seem to mostly occur between gene segments encoding equivalent domains, i.e. domains with the same positions in gene clusters from the same NRPS gene family (Tanabe *et al.*, 2004; Kurmayer and Gumpenberger, 2006; Papers I and II). We have however detected recombination events between *mcyB1* and *mcyC* within *Anabaena* and *Microcystis*; some of them resulting in the production of specific microcystin isoforms (Papers I and II). Recombination between *mcyB1* and *mcyC* has in some cases lead to replacement of a nearly entire A-domain (in *Anabaena* 18B6) and in others to replacement of a functionally important part of the domain in McyB1 (in *Microcystis* N-C 264), in both cases resulting in a change of function (i.e. amino acid activated) and the production of microcystin-RR. These results may indicate that in the investigated strains, homology-driven recombination between *mcyB1* and *mcyC* only leads to diversification of the McyB1 A-domain; putatively due to stronger functional constrains acting on the McyC A-domain.

Mutations introduced by recombination or other mechanisms have to spread in the populations through natural selection or genetic drift. Remodelling of microcystin synthetase genes has to result in a functional protein and not be deleterious to the host. The substrate specificity exhibited by the C-domain (Belshaw *et al*., 1999; Ehmann *et al*., 2000; Linne and Marahiel, 2000; Luo *et al*., 2002; Clugston *et al*., 2003; Samel *et al*., 2007) indicates that introduction of a novel A-domain only will result in production of a peptide if it activates an amino acid that can be accepted by neighboring C-domain. It has also been

shown that some artificial combinations of adenylation and condensation domains result in non-functional enzymes (Mootz *et al.*, 2000). All recombination events that lead to the replacement of A-domains shown by Mikalsen *et al.* (2003), Kurmayer *et al.* (2005), Fewer *et al.* (2007) and in Paper I and II, resulted in the incorporation of an amino acid with similar structure to the original one (in some *Planktothrix* strains, a Thr residue is incorporated instead of Ser by module McyA1) and or an amino acid already activated and condensed by the original McyB1 module (only Leu or Arg in modules which commonly incorporate Leu and Arg). This may indicate that C-domains probably restrict the selection of new constructs by only cooperating with suitable A-domains, but not necessary recombination as such.

Taken together, our results and other results (Mikalsen *et al*., 2003; Tanabe *et al*., 2004; Kurmayer *et al*., 2005; Kurmayer and Gumpenberger, 2006; Fewer *et al*., 2007) indicate that both intra- and intergenic recombination, point mutations and in-frame deletions combined with positive selection operating on specific codons reshape the *mcyBC* genes within *Anabaena*, *Microcystis* and *Planktothrix.* In this way, cyanobacterial strains might insure the production of those specific microcystin isoform(s) that are most advantageous in a given ecological setting.

4.2 Comparison of gene clusters encoding cyanopeptolin synthetases in Microcystis, Anabaena and Planktothrix

Cyanopeptolins (Figure 1B, chapter 1.2) are produced by strains from several cyanobacterial genera (for a review, see Welker and von Döhren, 2006). A cyanopeptolin synthetase gene cluster has been characterized in *Anabaena* (Figure 5, chapter 1.5.2) where a link between peptide and gene cluster has been established by knock-out of the operon (Rouhiainen *et al.*, 2000).

4.2.1 *In silico* methods for determining the substrate specificity of adenylation domains.

Different tools, such as phylogenetic analysis of sequence alignments and comparison of binding pocket signatures with previously defined signatures, were used to deduce the most likely amino acid activated by the A-domains of cyanopeptolin synthetases from *Microcystis* and *Planktothrix* (Paper IV and V). The limitation of both methods is the

restricted number of A-domain sequences and binding pocket signatures with experimentally verified, known amino acid selectivity. For example, the signature derived from the A3-domain of cyanopeptolin synthetase from *Planktothrix* corresponded to none of the defined signatures and the domain sequence clustered apart from the other A-domain sequences in the phylogenetic analysis, making it impossible to predict the activated amino acid based on sequence analysis alone (Paper V).

All cyanopeptolin synthetase A-domain sequences from *Microcystis* have binding pocket signatures that occur in the NRPS database or have been published before, making the deduction of a putative peptide structure easier. However, the phylogenetic analysis revealed some notable exceptions to the general picture of A-domains clustering largely according to the type of amino acid activated (Challis *et al*., 2000, Papers IV and V). The most striking was that the A-domain of McnE forms a well-supported clade together with Ile-activating A-domain sequences, despite having a binding site signature indicating that this domain activates Gln (Paper IV). The MS structure of the major peptide produced by this *Microcystis* strain indicates that, indeed, a Gln residue is present at the relevant position in the peptide, but formally, no causal link between the peptide and gene cluster was demonstrated, e.g. by knock-out studies. Gene knock-outs are difficult to establish in cyanobacteria, and an alternative would be to express the McnE A-domain and study its selectivity. If it can be verified that this domain, judged by phylogenetic analysis to be derived from an Ile-activating domain family, actually activates Gln (as suggested by the active site signature), it probably represents a case where point mutations rather than recombination have lead to altered substrate specificity.

4.2.2 Tailoring domains and enzymes

Is halogenase an ancestral feature in cyanopeptolin synthetase?

No examination has been performed to date regarding the evolutionary history of cyanopeptolin synthetase gene clusters, but it is reasonable to assume that cyanopeptolin synthetase has an ancient origin, similarly to that shown for microcystin synthetases (Rantala *et al.*, 2004) and that genes encoding cyanopeptolin synthetases were present in the last common ancestor of *Anabaena*, *Microcystis* and *Planktothrix*.

The presence of highly similar halogenase-encoding genes in the cyanopeptolin gene clusters of *Microcystis* and *Anabaena* (Paper IV, Rouhiainen *et al*., 2000) and lack of such a gene in *Planktothrix* (Paper V) raises the question as to the composition of the ancestral

cyanopeptolin gene cluster. Either the halogenase gene was present, and then lost in *Planktothrix* after diversification of the genera, or the halogenase was not present, in which case it must have been introduced later, but only in *Anabaena* and *Microcystis.* The halogenases are highly similar in the two *Anabaena* and *Microcystis* strains and show higher amino acid sequence identity than modules incorporating identical amino acids (Paper IV). This might indicate that the halogenase was introduced relatively late into the cyanopeptolin gene clusters of *Anabaena* and *Microcystis*, or suggest that stronger functional constraints acting on the halogenase compared to the other NRPS domains. However, sequence identity between cyanopeptolin halogenases and halogenases associated with other secondary metabolite biosynthetic gene clusters is strikingly low (Paper IV).

At first glance, the hypothesis of presence of halogenase in the ancestral cyanopeptolin gene cluster may seem the most likely, since it only involves gene loss in one genus. However, the high degree of similarity between halogenases of *Anabaena* and *Microcystis* may indicate a late introduction of this gene and a more complex evolution of these gene clusters. Further characterization of cyanopeptolin synthetase gene clusters from several strains from each genus (including *Microcystis* strains producing non-chlorinated cyanopeptolins, e.g. Martin *et al.*, 1993) is needed to resolve this issue.

The missing epimerization domain in *Microcystis*' cyanopeptolin synthetase

Nonribosomal peptides frequently contain D-amino acids. These residues are often activated as L-amino acids and then epimerized by epimerization (E) domain integrated into the module. When an amino acid is activated and then epimerized, it normally is bound to a specific subtype of T-domain (the T_E-domain), which is followed in the polypeptide by an E-domain. The stereochemistry of the C-terminal amino acid of the peptidyl chain has been reported to be an important element in substrate recognition at the C-domain donor site (Ehmann *et al.*, 2000; Luo *et al.*, 2002) (Figure. 3), and D-amino acid specific C-domains cluster together in phylogenetic analyses.

In Paper IV we report the unexpected juxtaposition of a T_E-domain and a D-amino acid condensing C-domain (C_D) without an intervening E-domain, indicating that a deletion of an E-domain-encoding segment has occurred near the end of *mcnA*. Phylogenetic analysis of cyanopeptolin synthetase A-domains suggests that the A-domain upstream of the T_E-domain activates L-Gln. Deletion of the E-domain should lead to unchanged

stereochemistry of the amino acid residue activated by upstream A-domain and might be expected to result in a non-functional cyanopeptolin synthetase, due to the inability of the C_D -domain to condense the L-Glu residue. Since this *Microcystis* strain produces cyanopeptolin, the synthetase must be functional, suggesting that the donor site in the D-amino acid condensing domain must have been altered to accept the L-isomer of Leu.

4.2.3 Formation of Ahp, an unusual amino acid found in cyanopeptolins

Characterization of cyanopeptolin gene clusters from three cyanobacterial genera has not resolved the pathway leading to formation of Ahp, the unusual amino acid at position 4 in cyanopeptolin ring. Rouhiainen *et al*. (2000) proposed that Gln may be the precursor of Ahp since the binding-pocket sequence shows the highest similarity to those activating Gln. Phylogenetic analyses of A-domain sequences from cyanobacterial NRPS gene clusters (Paper IV and V) revealed, however, that Ahp precursor-activating A-domains do not cluster together with Gln or Glu-activating domains, but form an independent clade. It should however be mentioned that no L-Glu activating A-domain was included in the phylogenetic analysis due lack of cyanobacterial L-Glu-activating A-domain sequences in the databases.

Rouhiainen and co-workers (2000) have proposed that ApdF, a protein encoded by a gene in the *apd* gene cluster, reduces enzyme-bound glutamate/glutamine to glutamic semialdehyde as a step in the formation of the Ahp residue. A gene encoding a protein homologous to ApdF is not present in the *mcn* and *oci* gene clusters, indicating that if such a protein is required for Ahp formation, it must be located elsewhere in the *Microcystis* and *Planktothrix* genomes. Another protein proposed to be involved in Ahp formation is the *N*-methyltransferase domain in module 5. Again, this domain is not present in the Mcn and Oci proteins. Clearly, more work is still needed to clearify the nature of the Ahp precursor and the reactions involved in Ahp formation.

4.2.4 Evolution of cyanopeptolin gene clusters

In paper IV and V, cyanopeptolin gene clusters were characterized from *Microcystis* and *Planktothrix*, making it for the first time possible to compare these operons in three different genera. Comparison of cyanopeptolin gene clusters reveals similar gene and domain arrangements, while the additional tailoring domains and the A-domain specificity vary. This suggests several independent recombination events that have resulted in loss and gain

of tailoring genes and altered amino acid specificity. No signs of recombination between cyanopeptolin gene clusters from different genera were found (paper V); again indicating the independent evolution within each genus, as suggested for the microcystin synthetase gene cluster (Rantala *et al*., 2004, Paper II). According to the original suggestion by Rudi *et al* (Rudi *et al*., 1998) and recently shown by whole genome analyses (Zhaxybayeva *et al*., 2006), recombination will occur more frequently between closely related strains. As for *mcy* genes (Mikalsen *et al*., 2003, Tanabe *et al*., 2004, Kurmayer and Gumpenberger, 2006, Paper I and II), recombination events in cyanopeptolin synthetase gene cluster thus might be detected by analyzing several strains within each genus, as recently done for *Planktothrix* (Rounge *et al*., in prep).

Cyanopeptolins and microcystins have co-existed in the *Microcystis, Anabaena* and *Planktothrix* groups, most likely over a long evolutionary period (Paper IV and V). Thus, a possible scenario would be that these two types of gene clusters containing the same basic building blocks (like A-, C- and T-domains) have recombined frequently during the evolutionary history. The phylogenetic trees of A- and C-domains presented in Paper IV and V show no evidence for such recombination between cyanopeptolin and microcystin synthetase gene clusters, indicating that these two classes of NRPS gene clusters have evolved independently. This may be an indication of distinct and non-overlapping functions and strong functional constraints.

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